AD-A176 551 MOLECULAR HETEROGENEITY OF PYRIDOXALATED-POLYMERIZED HEMOGLOBIN(U) LETTERMAN ARMY INST OF RESEARCH PRESIDIO OF SAN FRANCISCO CA G L MOORE ET AL. 04 DEC 86 1/1

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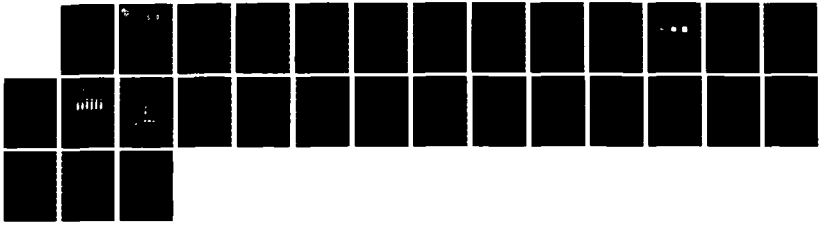

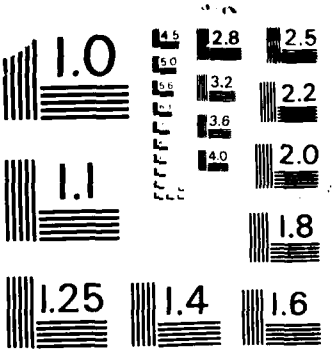

INSTITUTE REPORT NO. 227

MOLECULAR HETEROGENEITY OF PYRIDOXALATED-POLYMERIZED HEMOGLOBIN

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DECEMBER 1986

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: Institute Report No. 227	2. GOVT ACCESSION NO.: AD-A176 551
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE (and Subtitle): Molecular Heterogeneity of Pyridoxalated-Polymerized Hemoglobin	5. TYPE OF REPORT & PERIOD COVERED: Final
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): Gerald L. Mc , PhD; Mary E. Moore, BA; and Jerry A. Tillotson, MS	8. CONTRACT OR GRANT NUMBER(s):
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Blood Research Division, Letterman Army Institute of Research, Presidio of San Francisco, CA 94129-6800	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: WU-083
11. CONTROLLING OFFICE NAME AND ADDRESS:	12. REPORT DATE: 4 December 1986
	13. NUMBER OF PAGES: 23
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office): Medical Research and Development Command, Ft. Detrick, MD 21701-5012	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

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17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Polymerized-Pyridoxalated Hemoglobin, Electrophoresis, Blood Substitutes; Protein HPLC

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

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ABSTRACT

The molecular heterogeneity of solutions of pyridoxalated-polymerized hemoglobin has been examined in a stepwise manner by using high pressure liquid chromatography (HPLC), electrophoresis and incorporation of ^{14}C pyridoxal phosphate. Starting hemoglobin contained several major and several minor protein components, all of which appear to be pyridoxalated, some perhaps multiple times. The subsequent polymerization of this mixture with glutaraldehyde produces an extremely heterogeneous mixture of distinct protein species. We estimate the number of molecular species to be in the hundreds since there are too many to resolve by isoelectric focusing (IEF) or TSK-HPLC. However, SDS-PAGE electrophoresis reduced 75% of the hemoglobin to the same to the ~~same~~ monomer form as starting hemoglobin. This heterogeneity will add to the complexity of developing a modified hemoglobin as a "blood substitute".

ABSTRACT

The molecular heterogeneity of solutions of pyridoxalated-polymerized hemoglobin has been examined in a stepwise manner by using high pressure liquid chromatography (HPLC), electrophoresis and incorporation of ^{14}C pyridoxal phosphate. Starting hemoglobin contained several major and several minor protein components, all of which appear to be pyridoxalated, some perhaps multiple times. The subsequent polymerization of this mixture with glutaraldehyde produces an extremely heterogeneous mixture of distinct protein species. We estimate the number of molecular species to be in the hundreds since there are too many to resolve by isoelectric focusing (IEF) or TSK-HPLC. However, SDS-PAGE electrophoresis reduced 75% of the hemoglobin to the same to the same monomer form as starting hemoglobin. This heterogeneity will add to the complexity of developing a modified hemoglobin as a "blood substitute".

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MOLECULAR HETEROGENEITY OF PYRIDOXALATED-POLYMERIZED HEMOGLOBIN

Hemoglobin solution has been studied for many years as a possible oxygen-carrying resuscitation fluid (1,2). However, after hemoglobin is removed from the red cell it acquires two shortcomings as a "blood substitute." First, its oxygen binding is increased, as reflected by a drop in the P_{50} from 26±2 to 14±2 torr. Second, the free hemoglobin is quickly cleared from the vascular space (half disappearance time=3.5 hr) due to the renal clearance of dimer from the tetramer-dimer equilibrium. To overcome these limitations several workers have prepared a pyridoxalated-polymerized derivative of hemoglobin (3,4). The pyridoxalation step raises the P_{50} to near 20 torr, and the polymerization with glutaraldehyde forms nonspecific intra- and intermolecular cross-links which appear to retard the rate of renal clearance (3-5). Pyridoxalation must be done first to obtain an adequate P_{50} in the end product.

This report describes our efforts to define the molecular heterogeneity and distribution of the pyridoxalated-polymerized hemoglobin solution as prepared by DeVenuto and Zegna (3). We have defined the analytical techniques in the methods section of this paper. However, some of the applications of these techniques are mentioned in the results section to provide a clear understanding of the experimentation.

MATERIALS AND METHODS

Stroma-free hemoglobin (Hb) was prepared from outdated human red cells by either the crystallization procedure (6) or a modification of the Drabkin procedure (7). The Hb was adjusted to a concentration of 7 g/dl and modified (pyridoxalated and polymerized) by the procedure of DeVenuto and Zegna (3) in 25 to 100 ml volumes. This procedure consisted of deoxygenation of the Hb with nitrogen, addition of a 4 M excess of pyridoxal phosphate (pyr), followed by reduction with a 20 M excess of sodium borohydride and dialyzing against isotonic saline. The polymerization step consisted of concentrating the pyridoxalated hemoglobin (pyr-Hb) to 14 g/dl with Aquacide III, adding glutaraldehyde, and dialyzing against isotonic saline. The final product was centrifuged (30,000 x g, 1 hr) and filter-sterilized before analysis and/or frozen storage. One 25 ml batch of modified hemoglobin was prepared with the addition of 200 μCi of ^{14}C-pyridoxal phosphate, obtained from Amersham Corporation (Arlington Heights, IL).

Cellulose acetate electrophoresis was run using Gelman Sephrophore III strips in Tris-glycine-EDTA buffer, pH 9.2. The proteins were stained with Ponceau-S and analyzed by either a Beckman densitometer or by cutting out each band, dissolving it in acetic acid-methanol (1:1), and measuring the absorbance at 525 nm in a Gilford 240 spectrophotometer.

Sodium dodecyl sulfate (SDS) gel electrophoresis (SDS-PAGE) was performed in an LKB vertical gel system using a 4.2% stacking gel and a 10.2% resolving gel (8). Isoelectric focusing (IEF) was done in LKB PAG-Plates, pH 5.5-8.5 with LKB Multiphore or Ultraphore boxes (8). All gels were checked for the number of visible (red-"Hb") bands, then stained with the appropriate Coomasie blue dye (8). Autoradiography on certain gels containing ^{14}C proteins was done by air drying the gel and pressing it to an 8"x10" sheet of Kodak X-OMAT-AR x-ray film for exposure of 1 to 4 weeks. Standard x-ray developing techniques were used. Also, two SDS-PAGE gels containing the ^{14}C proteins were removed from their backing, cut into bands, oxidized in a Packard Model 306 oxidizer and counted in a Packard Tricarb 4530 counter. Data are reported as disintegrations per minute (DPM).

Pyridoxalated hemoglobin was analyzed by high pressure liquid chromatography (HPLC) on a Waters reverse-phase micro bondpak C-18 column using the procedure of Shelton *et al.* (9) and on a Brownlee CX-300 ion exchange column. The CX-300 column was eluted with a gradient of two buffers. Buffer A contained 0.04 M bis-tris plus 0.004 M KCN at pH 6.8. Buffer B was the same as buffer A with the addition of 0.2 M NaCl. The gradient program consisted of running for 5 min at 0% B, 5 min at 5% B, then increasing linearly to 50% B over 25 min. This was followed by 6 min at 100% B and a 5 min return to 0% B, all at a flow rate of 1.5 ml/min. Absorbance was monitored at either 289 or 410 nm. This HPLC work was done interchangeably on a Beckman Model 344, a Waters "peptide analyzer" or a Hewlett-Packard Model 1084 HPLC. Samples were diluted (usually to 1%) with buffer or water and filtered (0.4 μ) before injection.

Pyridoxalated-polymerized hemoglobin (pyr-poly-Hb) was examined by HPLC using an LKB TSK-4000 size exclusion column. The eluent was 0.1 M bis-tris containing 0.1 M NaCl, pH 6.8 at a flow rate of 0.7 or 1.0 ml/min. Absorbance was monitored at 280 or 410 nm. Protein molecular weight standards (Biorad Corp., Richmond, CA) were used to calibrate the column. These analyses were done on a Hewlett-Packard Model 1084 HPLC.

Hemoglobin content was measured by a micro Drabkin procedure (10), and methemoglobin by the method of Evelyn and Malloy (11). The P_{50} was determined by biotonometry (12). The oncotic pressure was measured with an IL Weil oncometer, Model 186.

RESULTS

Nonradioactive Preparations

After establishing our ability to perform the synthetic procedure (3), we prepared four 100 ml samples of pyr-poly-Hb to determine the reproducibility of the reaction sequence. In each preparation a small aliquot of the pyr-Hb was saved for analysis of the pyridoxalation step. The in vitro data are shown in Table 1 for the four preparations.

At the end of the polymerization step, the solutions contained a small amount of brown precipitate which was removed by centrifugation (30,000 x g, 1 hr). Amino acid analysis of this precipitate revealed an amino acid pattern essentially identical to that of hemoglobin.

The starting Hb, pyr-Hb and pyr-poly-Hb from each preparation were analyzed by cellulose acetate electrophoresis as shown in Figure 1. All four preparations were identical to each other and to a sample of pyr-poly-Hb donated to us by DeVenuto and Zegna (3). Pyr-Hb produced a distribution of about 60% of the protein to a more positive area of the gel than was seen for the starting Hb. The pyr-poly-Hb bands produced the same general pattern as pyr-Hb, except the bands were more diffuse and lacked clear separation.

The pyr-Hb samples were also analyzed on the CX-300 HPLC column. On this column the starting Hb produced a single peak. Analysis of the pyr-Hb samples produced two additional but poorly separated peaks, presumed to be pyridoxalated derivatives of Hb. These two additional peaks comprised 56 to 62% of the total heme protein.

Analysis of starting Hb on the HPLC reverse phase system (9) produced four major peaks: heme, beta chains, alpha chains, and undefined nonprotein material (Fig. 2). HPLC of the pyr-Hb samples revealed a splitting of the beta chain peak into three peaks of approximate total area of the original Hb beta peak. The one beta peak (of pyr-Hb samples) that had a retention time similar to that of

TABLE I

Solution Properties (Ranges)

Sample	% MetHb*	P50 (mm)	Oncotic Pressure (mm)
Starting Hb	3 to 5	12 to 14	21 at 7% Hb
Pyr-Hb	2 to 7	25 to 29	----------
Pyr-Poly-Hb	10 to 15	16 to 19	16 to 17 at 14% Hb

* Percent of total protein

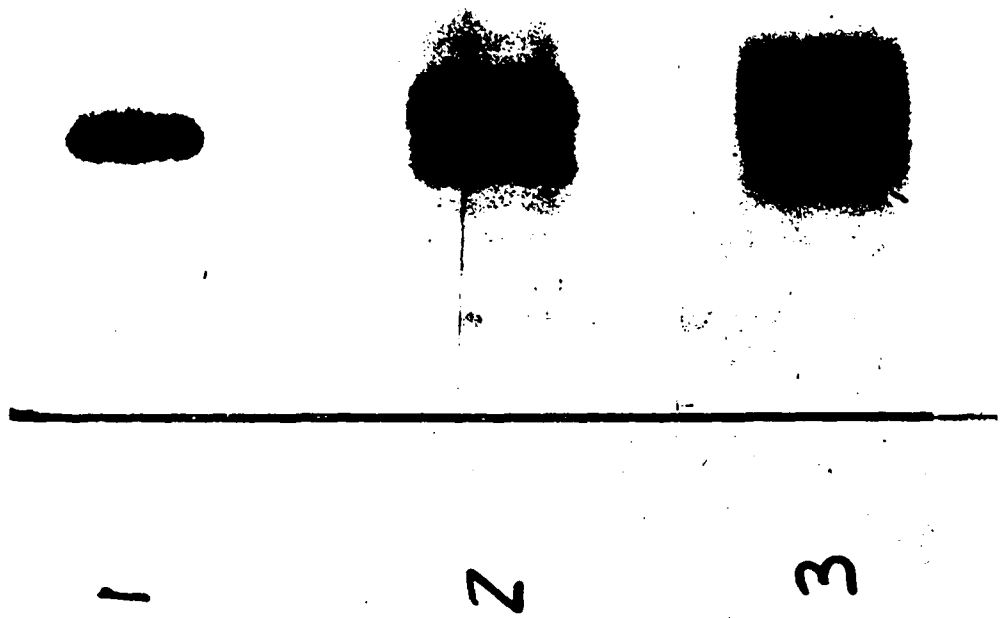

Figure 1

Cellulose acetate electrophoresis of (1) starting Hb, (2) pyr-Hb and (3) pyr-poly-Hb. Cathode is to the right, solid line is the point of application.

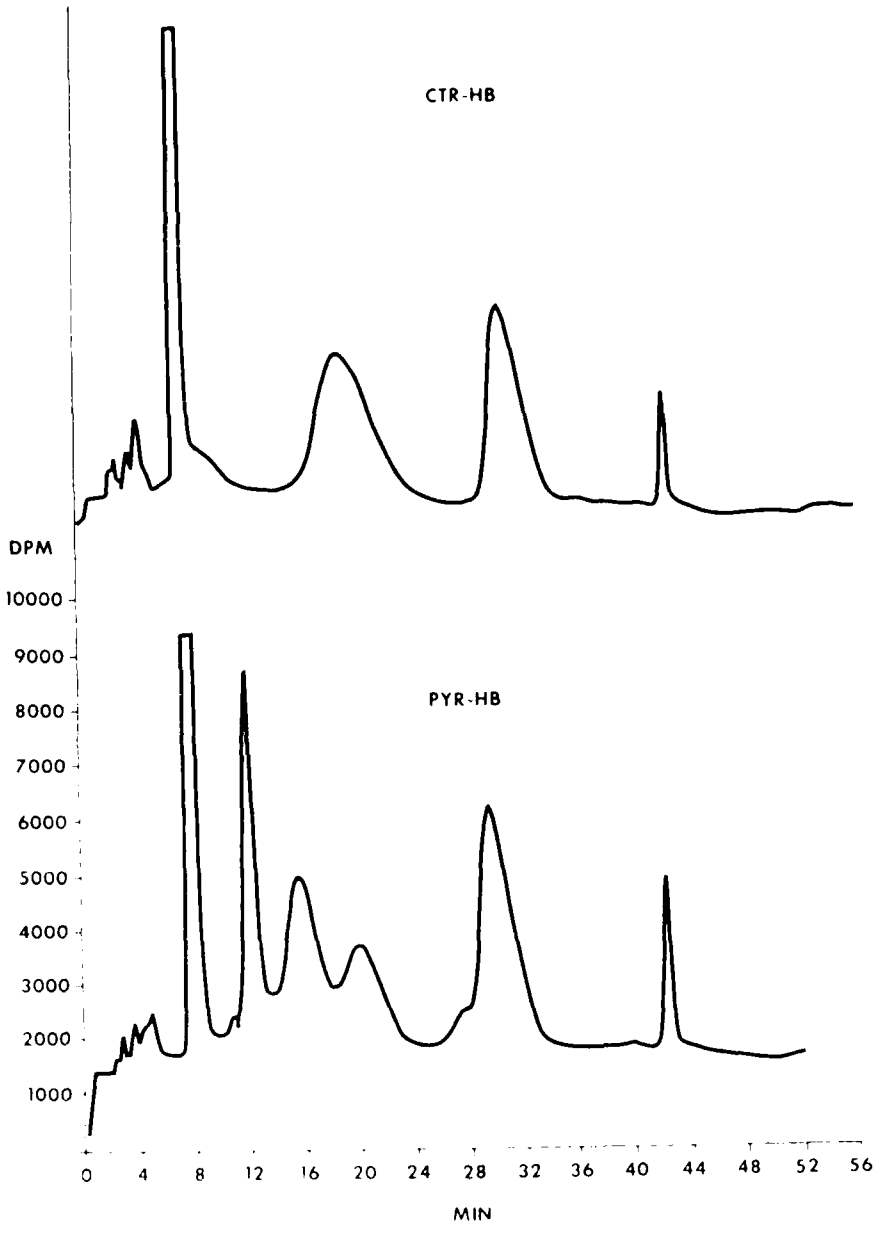

Figure 2

Reverse phase HPLC separation of starting Ctr-Hb (top) and pyr-Hb (bottom). Components of top chromatogram (with retention times) is as follows: contaminants (2-6 min), heme (8 min), unreacted β chain (18 min), α chain (30 min), and undefined nonprotein material (42 min).

the Hb control represented 36 to 50% of the total "beta peaks" area. The two "modified beta" peaks were about equal in total area. The incomplete separation of the "beta peaks" made exact quantitation of their relative amounts somewhat subjective.

The starting Hb and the pyr-poly-Hb samples were run on a TSK-4000 column (Fig. 3). Many attempts were made to improve the peak separation of pyr-poly-Hb samples, but none were superior to the results shown in Figure 3. All the modified Hb samples (including the gift from F. DeVenuto) gave identical chromatograms. In the modified Hb absorbance scans, the area which corresponded to the area seen for starting Hb represented 70 to 80% of the total area.

One sample of pyr-Hb was repyridoxalated by the same procedure used initially. Subsequent HPLC on the CX-300 column showed an increase of 15% in the total pyridoxalated peak area. There was no change seen in the P_{50} (29 mm) or the metHb (2% total Hb).

Radioactive Preparation

One 25 ml sample of pyr-poly-Hb was prepared using ^{14}C pyridoxal phosphate in an attempt to clarify earlier results with "cold"-pyr. An aliquot (5 ml) of the ^{14}C was retained for analysis. Isoelectric focusing of these samples is shown in Figure 4. The results are similar to those obtained for the nonradioactive preparation (data not shown). The anode (pH 8.5) is at the bottom and the cathode (pH 5.5) is at the top of the figure. Lane 1 is the starting Hb from the gel. Lanes 2B, 3B, and 4B are stained gel bands for the pyr-Hb and two concentrations of pyr-poly-Hb, respectively. The "A" lanes are corresponding autoradiograms printed from the x-ray film and properly aligned with the corresponding gel bands. Every stained band in the gel contained a radioactive pyridoxal group. A separate print made from the x-ray film was exposed to reveal the presence of minor components (loss of clarity of the major components). This modified print (not shown) revealed 15 to 18 additional bands with isoelectric points lower than the major bands that are shown in Figure 4.

The ^{14}C samples were also analyzed by SDS-PAGE which dissociated the proteins into monomeric subunits and separated them by molecular weight (Figure 5). Starting Hb and pyr-Hb showed similar results, but there was a slight spreading of the low M.W. band (approximately 17,000) seen in the pyr-Hb sample. The pyr-poly-Hb sample showed evidence of higher-molecular-weight forms (30,000 and 42,000 daltons).

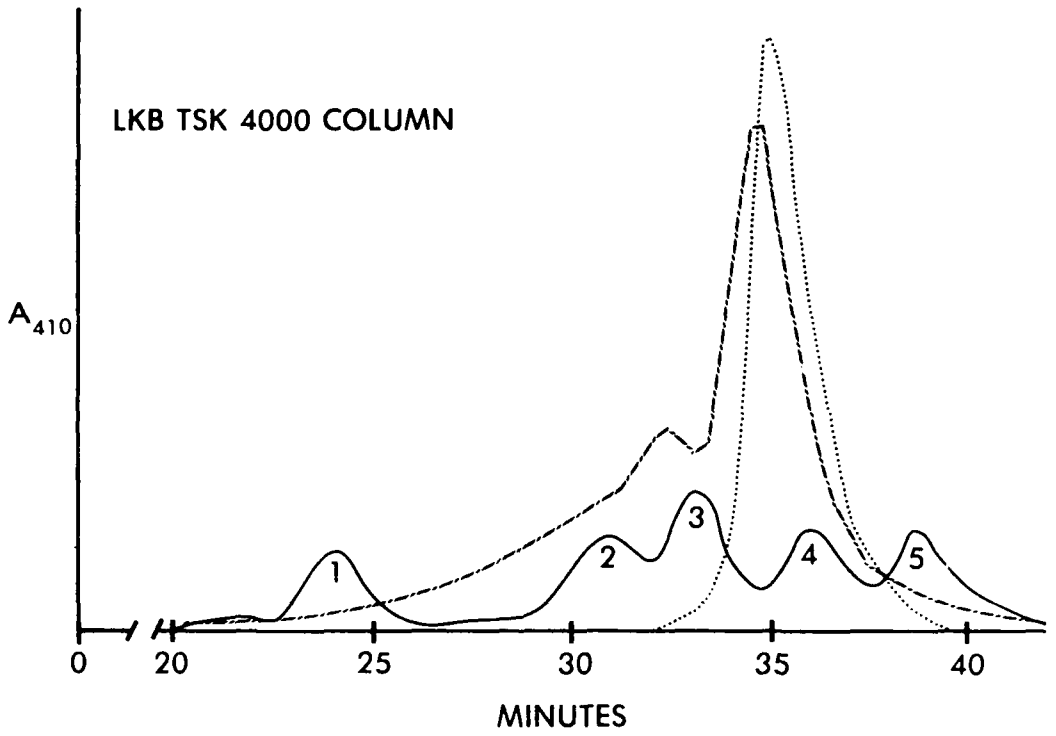

Figure 3

TSK 4000 HPLC overlay of 3 runs. Pyr-poly-Hb is the dot-dashed line; starting Hb, the dotted line; and molecular-weight standards, the solid line. Standards (1 to 5) are 670, 158, 44, 17 and 13 kilodaltons.

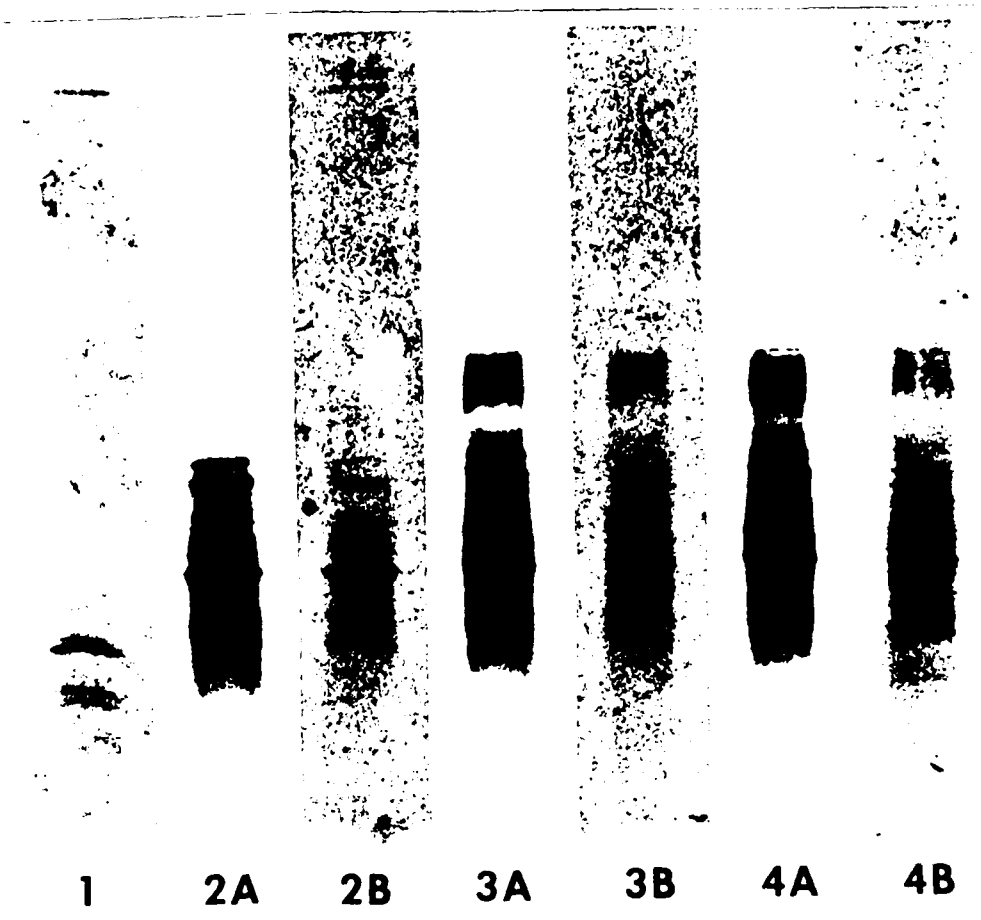

Figure 4

Autoradiography (A) and IEF (B) of starting Hb (1), pyr-Hb (2A,2B), and two concentrations of pyr-poly-Hb (3A,3B,4A,4B). Cathode (pH 5.5) is at top of figure.

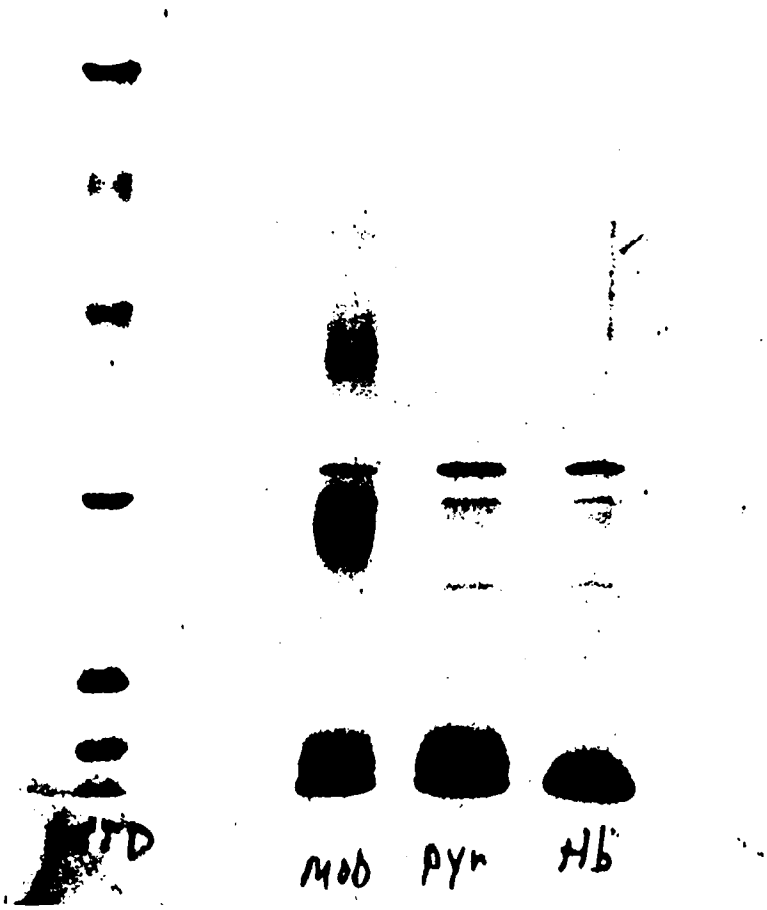

Figure 5

SDS PAGE electrophoresis of protein standards, pyr-poly-Hb, pyr-Hb, and starting Hb. Standards are (bottom-top) 14, 21, 30, 43, 68, and 94 kilodaltons.

A similar SDS-PAGE lane was evaluated by oxidation and counting (DPM); the results showed 80% monomer, 12% dimer, 4% trimer and 1% tetramer.

The pyr-Hb intermediate was also evaluated by HPLC using the CX-300 and the reverse phase columns. Figure 6 shows the results from the CX-300 columns where the radioactivity (from 1 ml fractions) is plotted as the solid line and the absorbance as the short dashed line. An overlay of the position of the starting Hb is shown as the long dashed line. The peaks for absorbance and radioactivity correspond closely for the peaks at 27 and 33 ml, which are the two new major peaks produced by pyridoxalation. However, 45% of the total radioactivity is seen in the first 10 ml of the separation, which corresponds to only 19% of the total absorbance. Some of this early eluting material is thought to be nonheme protein since a small peak is seen at 5 ml with the starting Hb sample. In this run the absorbance peak at 40 ml consists of 49% of the total Hb (23 to 46 ml) absorbance area. This separation was done with a different CX-300 column and produced better peak separation than was seen with the earlier non-^{14}C samples.

The reverse phase HPLC is shown in Figure 7. The dotted line is absorbance and the solid line is radioactivity. The radioactivity appears in absorbance peaks at 11 and 17 min, corresponding to the modified beta chains. The integrated absorbance area of these two peaks is almost identical. The peak at 11 min contains almost twice the DPM (14300 versus 8700) as the 17 min peak. There is also evidence of pyridoxalation occurring on the alpha chain as seen by radioactive shoulder at 29 min. The 28-29 min peak is due to pyridoxalation and is positioned left of the alpha peak, just as pyridoxalation beta peaks shift to the left of unreacted beta. In addition, if the area of this peak plus the area of the alpha peak are divided by the sum of the areas of the beta peaks, the usual ratio between alpha and beta peak areas in unreacted Hb is obtained (0.66). The pyridoxalated peak at 44 min (undefined material) is not protein in nature. When collected, dried, hydrolyzed with HCl, and reacted with ninhydrin, there is no color reaction. Additional evidence on the nature of these peaks was obtained by ratio scans of typical runs using 220/260 and 220/280 nm ratios. The peaks at 8 and 44 min behaved differently than the other peaks. Scans of each peak showed the expected minimal absorption for proteins at higher wavelengths, relative to 220 nm. Peaks at 8 and 44 min appeared typical of highly conjugated organic molecules. Shelton et al. (9) feels that this peak is a "purge peak" of contaminants obtained when the gradient reaches its maximum percent of solvent B. The distribution of radioactivity is 13% on alpha chains, 11% on undefined material, and the remainder on the beta chains (at least two separate products).

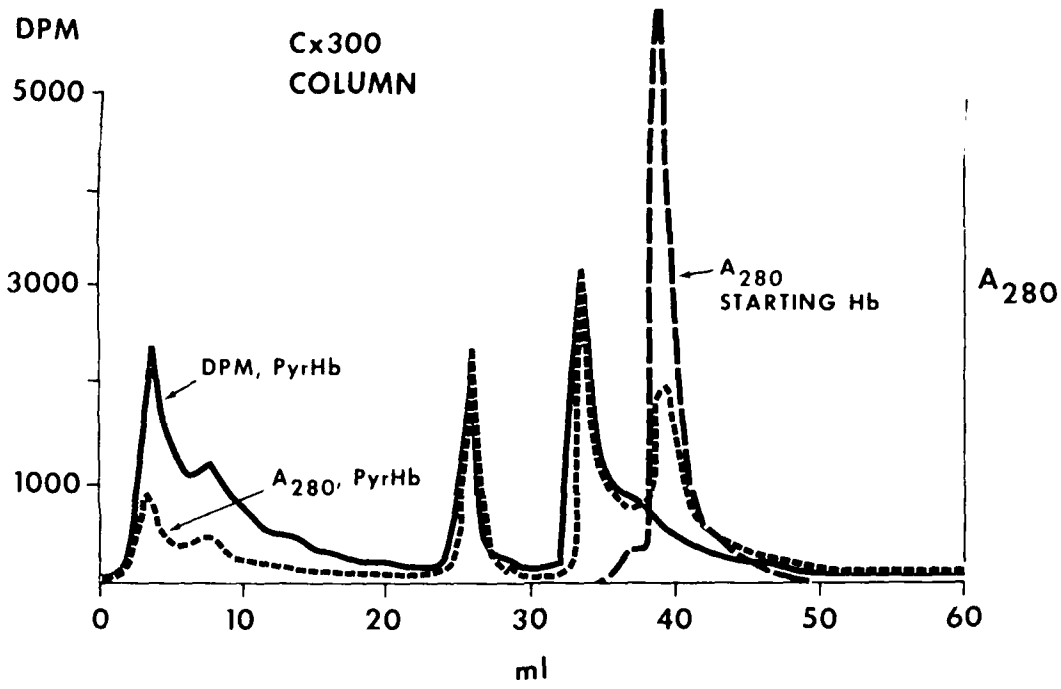

Figure 6

CX-300 HPLC of ^{14}C-pyr-Hb with an overlay (long dashed line) of starting Hb. Solid line is radioactivity and dotted line is A_{280}. DPM times corrected to negate the holdup volume between UV detector and fraction collector.

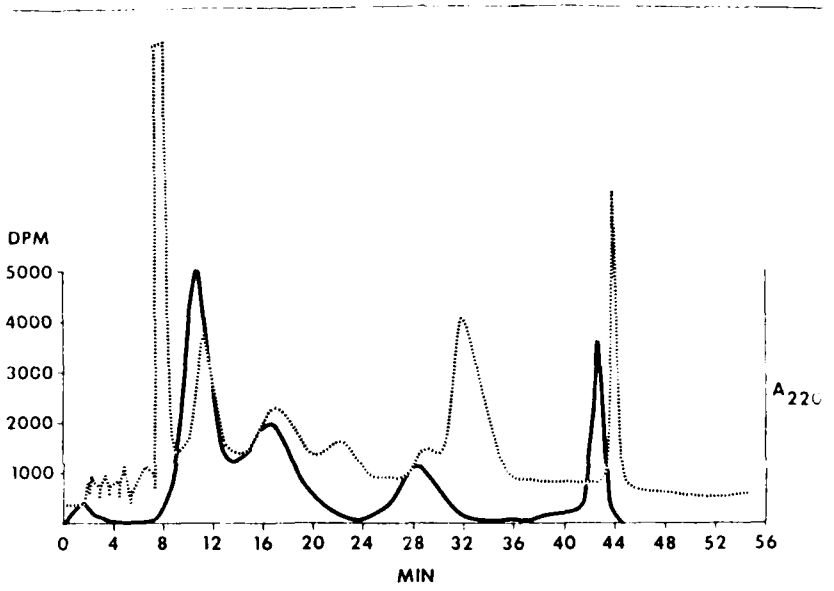

Figure 7

Reverse-phase HPLC of ^{14}C-pyr-Hb. Solid line is DPM, and dotted line is A_{220}. In this run the DPM elution time was corrected for holdup volume between UV detector and fraction collector.

The ^{14}C-pyr-poly-Hb was evaluated on the TSK-4000 HPLC column. The absorbance results (Fig. 8) are similar to those in Figure 3; however, the DPM trace shows a rise in the "tetramer" region. The figure is divided into two parts at the 44 kd point. Part I contains 22% of the absorbance area and 26% of the DPM, while Part II contains 78% of the absorbance and 74% of the DPM.

DISCUSSION

The pyridoxalation and polymerization of stroma-free Hb produces a solution with adequate P_{50} and intravascular retention to make it an attractive candidate for use as a resuscitation fluid, or "blood substitute" (3,4). Polymerization with glutaraldehyde also lowers the oncotic pressure so that a 14 g/dl solution is iso-oncotic with 7 g/dl starting Hb or human plasma. This polymerization allows one to double the concentration of the oxygen carrying protein. There have been no studies done to determine if these reactions (pyridoxalation and polymerization) affect the toxicity of hemoglobin solutions. However, it appears that these reactions dramatically increase the molecular heterogeneity of the protein solution, perhaps increasing the potential for toxicity, while definitely compounding the problems of characterization and quality control.

The starting hemoglobin solution is heterogeneous. Although the predominant form of Hb in solution is A1, there are also measurable amounts of other forms including A2, A1C, etc., plus various oxidized and ligand bound derivatives. If Hb solution is made in large batches from pooled donors, the presence of genetically different hemoglobins is also probable. Hemoglobin solutions also contain lesser quantities (5%) of numerous nonheme proteins that are difficult to separate from the Hb (13). This heterogeneity is shown in the electrophoresis and IEF data of Figures 1, 4, and 5. Our initial studies were done with Hb prepared by crystallization (6), but due to endotoxin contamination, we switched to using Hb prepared by a modified Drabkin procedure (7). These two preparations of Hb solution had identical physiochemical properties and gave identical separations on all types of electrophoresis and HPLC. Thus for the purposes of our study we considered the two sources identical. Each contained several protein species.

The pyridoxalation reaction used by DeVenuto and Zegna (3) is a slight modification of Benesch's procedure (5). DeVenuto and Zegna increased the molar ratio of pyridoxal phosphate:Hb from 2:1 to 4:1 to obtain a higher P_{50} in the final product. DeVenuto and Zegna report (3) a yield of 80% pyridoxalation, which is similar to that seen by

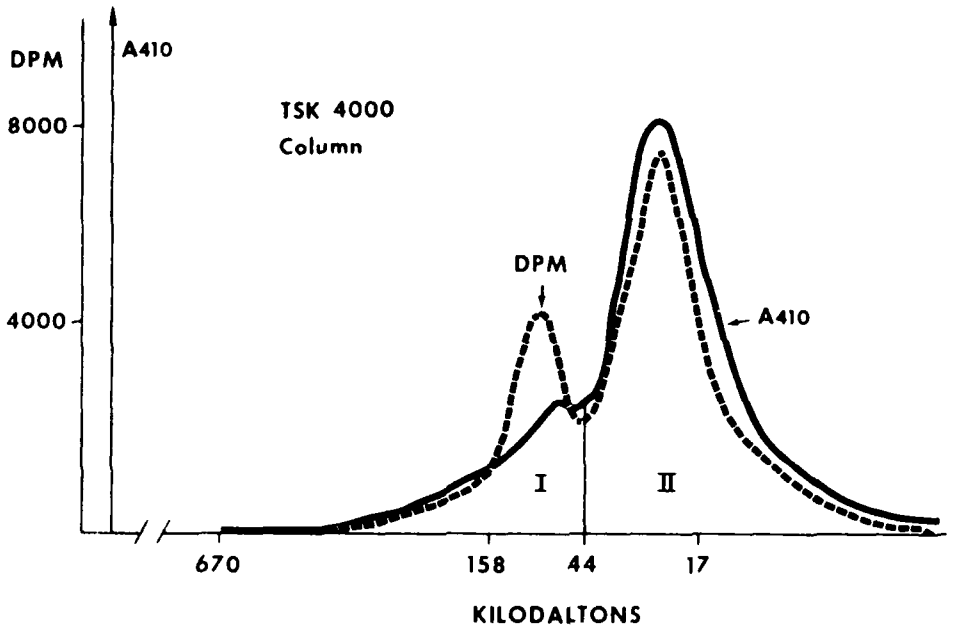

Figure 8

TSK 4000 HPLC of ^{14}C-pyr-poly-Hb. Solid line is A_{410} and dotted line is DPM. The integrator divided the chromatogram into two "peaks" at the 44 kd point.

Benesch (5); however, it must be remembered that this is the initial yield under deoxy conditions. Benesch et al. (5,14)ml reported that the initial reaction was a mixture of 80% mono-pyr at the val 1 and 10% symmetric di-pyr at the β val 1,lys 82. When these products are exposed to oxygen there is a partial dissociation into dimers with the equilibrium driven reassociation favoring symmetric tetramers. Benesch (14) thus obtained a final equilibrium mixture of 4% tetra-pyr $\alpha_2\beta$ (1 val 1 lys)$_2$, 45% di-pyr $\alpha_2\beta$ (1 val)$_2$, 6% $\alpha_2\beta\beta$ (1 val) and 45% "unreacted" tetramer.

Our data for the nonradioactive reactions analyzed by cellulose acetate electrophoresis and CX-300 HPLC seem to confirm the Benesch results in that we obtained a mixture of hemoglobins which is about 40 to 45% nonreacted. However, when we used the ^{14}C-pyr and separated the monomers by reverse phase HPLC, we obtained different results. On this column the Benesch data would yield 100% free α 48% free β, 48% mono-pyr β, and 4% di-pyr β. We obtained about 40 to 45% unreacted β, but an almost equal distribution of what appears to be 1x and 2x pyr beta chains. This result would imply a higher percentage of dipyridoxalation in the initial deoxy reaction, perhaps due to the doubling of the pyr:Hb ratio, while the percent of unreacted Hb stayed relatively constant. The data from our CX-300 column support the concept of two ^{14}C-pyr modified hemoglobins of relatively equal concentration. It is difficult to be more specific with the CX-300 column since we do not know if the fundamental separated unit of starting Hb is the tetramer, the αβ dimer, or monomer with no resolution between unmodified α and β chains. We observed with interest that about 13% of our pyridoxalation occurred on the α chain and that a minor nonprotein component was also pyridoxalated.

The results of isoelectric focusing of the ^{14}C proteins indicated that pyridoxalation occurs, perhaps, at multiple amine sites on most, if not all, of the minor hemoglobins, contaminating proteins and contaminating organic materials of the starting solution. Careful analysis of the autoradiogram for ^{14}C-pyr-Hb reveals 6 to 7 major species and 20 to 30 minor components. Most of these minor components were not detectable by staining the gel with Coomasie blue.

When one sample of pyr-Hb (non-^{14}C) was repyridoxalated, the amount of free Hb (as seen by CX-300 HPLC) dropped almost by half. This finding supports the Benesch (14) concept of equilibrium rearrangement of pyridoxalated species. Our experiment showed that the total amount of pyridoxalated Hb could be increased by repyridoxalation, however, this increase did not result in an increase in the P_{50} of the solution.

The polymerization of the proteins with glutaraldehyde in the pyr-Hb solution in nonspecific. The aldehyde groups on each end of the glutaraldehyde molecule can react with any free amino group in solution. This makes most of the free N-terminal groups and lysyl side chains candidates for reaction. Many monomeric reactions may occur with the subsequent formation of many cross-linked products within the same molecule (intramolecular) or adjacent molecules (intermolecular). Some monomeric glutaraldehyde reactions may undergo oxidation at the free aldehyde end or react with a low-molecular-weight contaminant of the solution. Certain nonheme proteins in the solution may be more reactive than Hb. Intermolecular cross-linking of Hb could be trimeric or tetrameric, as well as dimeric. The net result of all these nonspecific reaction possibilities is the formation of a great number of structurally distinct monomeric and cross-linked species. In fact, the mathematical law of combinations predicts thousands of possible, unique molecular combinations. Habeeb and Hiramoto (15) have examined the reaction of glutaraldehyde with ovalbumin and bovine serum albumin. They found that the reaction was primarily with lysine side chains, but also occurred with the reactive nucleophiles in the side chains of cysteine, tyrosine and histidine residues. They obtained a mixture of intra- and intermolecular cross-linked products, the ratios of which were dependent on the relative concentrations of protein and glutaraldehyde. They also confirmed that a mixture of starting proteins could be cross-linked to each other. The kinetic implications of the resultant product mixture has been shown in a theoretical model which was tested on the glutaraldehyde cross-linking of lactate dehydrogenase (16). Reichlin and Visoff (17) have shown that the immunological activity of cytochrome-c in rabbits is dramatically increased after glutaraldehyde cross-linking of the cytochrome-c in several animal species. The situation is further compounded by the possibility of dimer formation at equilibrium in those species in which two associated $\alpha\beta$ dimers are not intramolecularly cross-linked. This could produce equilibrium rearrangement of "cross-linked species" in a manner similar to that observed by Benesch et al. (14) for pyr-Hb solution. Figure 9 schematizes a few of the theoretical possibilities for this type of reaction. One net result of this type of process would be the formation of a large reassociation of uncross-linked or monoreacted $\alpha\beta$ dimers originally associated with cross-linked tetramers.

All of our data for the separation of pyr-poly-Hb solutions support the validity of the above arguments for the gross heterogeneity produced by nonspecific cross-linking. The cellulose acetate electrophoresis pattern seen in Figure 1 is a single large stripe. The IEF gel in Figure 4 is also one large stripe which overlays the heavier bands identical with starting Hb and pyr-Hb. The

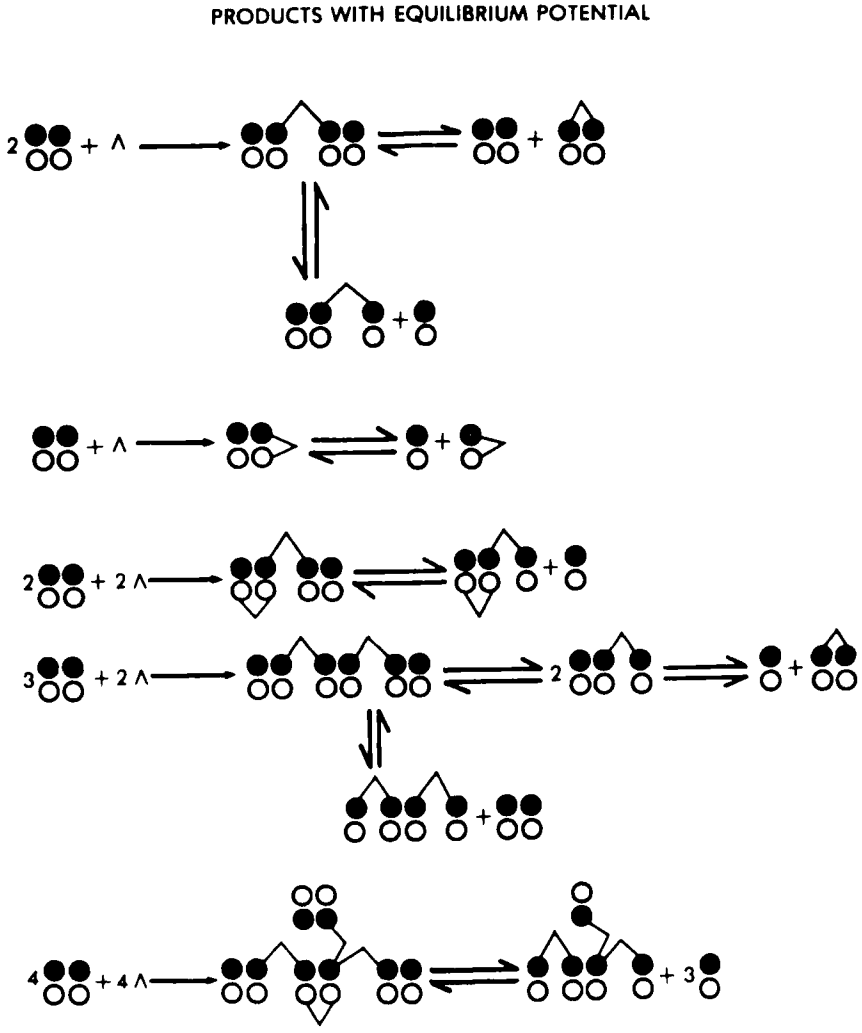

Figure 9

Some theoretical Hb intermolecular crosslinked products with equilibrium rearrangement potential. Filled versus open circles distinguish between and monomers. The inverted V represents the glutaraldehyde cross-link.

lack of focusing by pyr-poly solutions indicates an almost continuous spectrum of isoelectric points (pI) among the multitude of components of this mixture. The IEF can separate proteins that differ from each other in pI by as little as 0.02 pH units (18). The stained IEF band covered 1.5 pH units and should have been capable of resolving 50-75 component species. The lack of resolution in the IEF implies that a greater number of different electronic species are present. Identical reactions on two different sites on a protein may introduce minor changes in pI due to subsequent steric shielding of adjacent local groups from interaction with the aqueous environment. A similar, almost continuous, distribution of molecular-weight ranges was seen in the TSK-4000 HPLC. The absorbance shows a small but continuous rise in the curve through the molecular-weight ranges of 600 to 60 K dalton, with the suggestion of a peak at 45 to 60 K dalton. A large peak in the monomer-dimer range has the same retention characteristics as unreacted Hb and encompasses 75 to 80% of the total protein. This suggests that the bulk of the initial reactions between pyr-Hb and glutaraldehyde are either monomeric species, intramolecular cross-links, or dimeric intermolecular cross-links. This conclusion is supported by the SDS-PAGE data where 80% of the protein appeared as monomer, 12% dimer, and 4% as trimer.

We conclude that the pyr-poly-Hb solution is an extremely heterogeneous solution which contains perhaps hundreds of molecularly unique species instead of several simple oligomers as originally proposed (3,4). This condition arises from the heterogeneity of the starting Hb, the fact that every protein appears to be pyridoxalated (perhaps multiple times), the huge number of products formed by the nonspecific glutaraldehyde reaction, and the possible formation of new species by the dissociation-reassociation equilibrium phenomena. Heterogeneity does not exclude the development of pyr-poly-Hb as a "blood substitute." But this heterogeneity does compound the difficulty of optimizing and controlling the quality of such solutions, since it will be almost impossible to isolate those particular species in the mixture that are most efficacious or perhaps are yielding side reactions. This fact forces one to study only the solution's bulk properties to which many different molecular species have varying contributions. This complication does not preclude developing modified Hb solutions for clinical use as a blood substitute since mixtures such as Plasmanate and dextran are heterogeneous mixtures. However, the heterogeneity will compound the difficulty of scientifically understanding the solution and developing adequate quality control of its preparation.

ACKNOWLEDGEMENT

We wish to acknowledge the help of many co-workers in this study: F. DeVenuto, PhD, and A. Zegna for supplying crystalloid starting Hb solution, guidance on the pyridoxalation-polymerization reactions, and a reference sample of pyr-poly-Hb; R. Bolin, MD, for supplying modified Drabkin preparation of Hb; R. Tye, MD, for amino acid analysis; and C. Peek, D. Hankins, and K. Trimble for help with the P_{50} analysis and some of the HPLC work.

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